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RESPIRATION AND PHOTOSYNTHESIS IN *CONVOLUTA ROSCOFFENSIS* GRAFF, INFECTED WITH VARIOUS SYMBIONTS [1]

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Measurement of photosynthesis and respiration in algal-invertebrate symbiosis has been used as a means of assessing the functional significance of these associations (for review, see Droop, 1963). Early studies made extensive use of chemical methods for the determination of oxygen produced by the algal partner (Yonge, Yonge, and Nicholls, 1932). The use of polarographic oxygen electrodes offers advantages over the chemical method (Kanwisher, 1959) and this technique has been applied to the symbiosis in hermatypic corals (Kanwisher and Wainwright, 1967; Roffman, 1968), the acoel *Amphiscolops* (Taylor, 1971a) and the sea slug *Tridachia* (Taylor, 1971b). Studies on the symbiosis between the marine flatworm *Convoluta roscoffensis* (Graff) and its green algal symbiont *Platymonas convolutae*, Parke and Manton would be useful for comparative purposes, since the animal appears totally dependent on the algae for its nutritional needs.

The possibility of culturing *C. roscoffensis* in the laboratory (Dorey, 1965), permitted an analysis of the specificity of the symbiosis and led to the finding that a species of another genus, *Prasinocladus marinus* and other *Prasinocladus*-like alge could establish a successful symbiosis with *C. roscoffensis* (Provasoli, Yamasu, and Manton, 1968). The present study attempts to determine the differences in photosynthetic ability of these natural and experimental symbioses.

Materials and Methods

Worm and symbionts

Worms used in these experiments belong to three strains collected at Jersey, Roscoff and Hendaye. Continuous cultures were grown in seawater + 2 ml/100 Provasoli's ES enrichment (1968) at 15° C with alternating day/night (14 h/10 h) cycles. The cultures received 500 ft-c illumination provided by cool white fluorescent tubes placed 15–30 cm from culture dishes. Detailed information on the cultivation and maintenance of *C. roscoffensis* in the laboratory will be published elsewhere.

The photosynthetic capabilities of worms infected with different symbionts were examined. These included strains of *P. convolutae* designated as cultures S 6

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374, W 12 (see Provasoli, Yamasu and Manton, 1968) and Hendaye (from Hendaye worms) and unnatural symbionts, *Prasinocladus marinus* (Plymouth 308), Prasinocladus sp. (S 47), (Parke and Manton, 1965), and *Tetraselmis verrucosa* Butcher (1959).

Measurement of O_2 production and consumption

A Clark polarographic oxygen electrode (Yellow Springs Instrument Co.) connected to a chart recorded (Varian G-1000) was used according to the methods given by Kanwisher (1959). During experiments the electrode was immersed in the medium by inserting it into the side of the glass chamber illustrated in Figure 1. Temperature was maintained at 15° C by circulating constant temperature water (Haake FE circulator) through the outer jacket of the chamber. Illumination was provided by a Sylvania Tungsten Halogen Quartz DWE 150

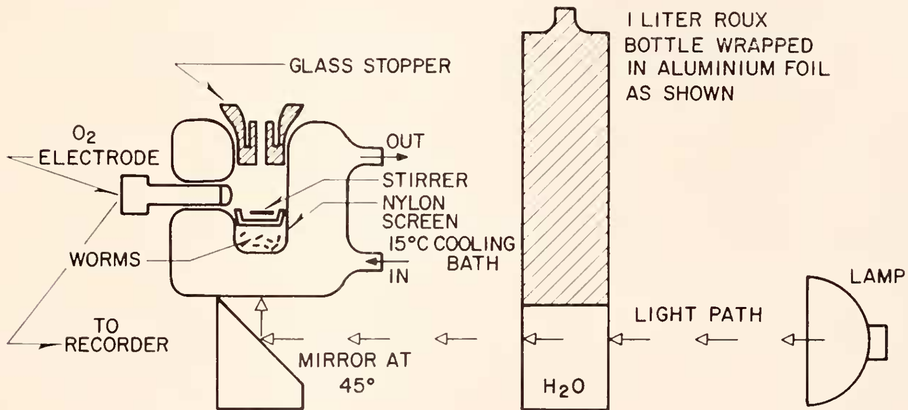

FIGURE 1. Diagrammatic section through the apparatus used for the measurement of oxygen production and consumption. Oxygen determinations are made by a Clark type polarographic electrode (Yellow Springs Instrument Co.). For further details, see text.

watt projection lamp connected to a rheostat to give variable light intensities. During experiments light was reflected through the bottom of the chamber by an angled mirror. Side illumination was excluded by the aluminum foil covering part of a cooling water bottle (Fig. 1). Intensity at various rheostat settings was measured in foot-candles with a photometer. Dark periods were achieved by switching off the light and covering the chamber with a double thickness of black cloth.

For each experiment 10–20 adult worms of uniform size and condition were placed in the well of the chamber which, when stoppered, contained 1.5 ml sterile medium consisting of charcoal treated seawater with 2.0 ml/100 ES enrichment and 0.02 g% $NaHCO_3$. A tight fitting plastic ring with a screen of nylon plankton net was inserted over the worms to confine them to the lower portion of the well and to form a platform for the magnetic stirring bar used to circulate the medium (Fig. 1). One half of the screen's open area (a center stripe) was

painted with epoxy glue to reduce the force of water flow in the lower part of the well. Strong flow (even at slowest stirring speeds) disintegrated some worms during these experiments. Following these procedures, the well was covered with a glass stopper and the system allowed to equilibrate for 2–3 minutes before beginning measurements. Experiments usually consisted of consecutive light and dark periods lasting 15 minutes each. Longer time periods (30–60 minutes) were employed for the determination of light compensation points for each symbiont type. At the termination of each experiment, worms were removed from the well, placed in sterile medium and returned to the incubators for 24 hr with a normal light/dark cycle. These were then examined for deaths or abnormalities resulting from the experimental procedure. If none were found, measurements of volume and chlorophyll a content were carried out as described below.

TABLE I

Average photosynthesis/respiration (P/R) values obtained at 1000 foot candles. Each cycle represents one 15 min light period followed by one 10 min dark period. Consecutive cycles were performed on the same group of 10 worms without a break in recording. Calculation of P/R is according to Roffman (1968),

i.e., $\frac{P}{R} = \frac{(P + R)}{R}$

Host symbiont	No. of experiments	Cycle	Average net photo µl O_2/hour	Average respiration µl O_2 hour	Average $\frac{P + R}{R}$
Jersey/S 6	6	I	1.48	1.71	1.87
		II	1.70	1.72	1.99
Roscoff/W 12	6	I	2.42	2.64	1.93
		II	2.41	2.68	1.91
Jersey/308	6	I	3.23	3.08	2.05
		II	2.78	3.19	1.87
Jersey/*T. verrucosa*	6	I	1.60	1.88	1.85
		II	1.62	1.84	1.90

Rates of photosynthesis and respiration were calculated by drawing tangents to the recorded tracing at the beginning of the light (photosynthetic rate = P) and dark periods (respiration rate = R) and extending these from 0–100%. The tracing of O_2 production or consumption for 15 min periods was almost always a straight line. Only when the light period lasted longer (~ ½ hour), was a steady decline in O_2 production observed. Gross photosynthesis was taken as the sum of P + R and the ratio gross photosynthesis/respiration was calculated from (P + R)/R = P/R (Roffman, 1968).

Average values derived from these calculations are presented in Table I. In other experiments, the values for net photosynthesis and dark respiration were related to worm volume and chlorophyll a content. Examples of these experiments are presented in Table II. Repeat experiments were qualitatively the same for each individual symbiont type studied. Minor variations in the net rate of photo-

synthesis between different symbiont types were found to lie within the range of variation encountered with the techniques used.

Worm volume and chlorophyll a content

The total volume of worms in each experiment was determined by centrifugation in capillary tubes. After 24 hr (see above), the worms were pipetted into a micro-hematocrit tube (Clay Adams, Inc., 0.55 mm diameter) open at two ends. One end was sealed with vinyl plastic putty and the capillary was centrifuged for 7 minutes at 12,000 rpm. The length of packed worms was then measured to the nearest 0.1 mm against a scale viewed in a microscope and the volume calculated.

Packed worms were pushed out of the capillary onto a piece of glass filter paper, homogenized with 2 ml of 90/10 acetone/water in a glass tissue homogenizer, and the resulting liquid placed in a conical centrifuge tube. The homogenizer was washed with three 1 ml portions of the acetone/water mix and this added to the extract. After adjusting the volume to 5 ml, the tube was stoppered and covered with Parafilm and aluminum foil. This was stored under refrigeration for 24 hr. The precipitate was then resuspended and centrifuged. The supernatant was

TABLE II

Oxygen production at varying light intensities

Host	Symbiont	Light (ft-c)	Net photo. $\mu l\ O_2/h$ / mm^3 worm	μg chl.a / mm^3 worm	Net p. / chl.a	Host	Symbiont	Net photo. $\mu l\ O_2/h$ / mm^3 worm	μg chl.a / mm^3 worm	Net p. / chl.a
Hendaye	own	500	1.07	0.76	1.42	Roscoff	*Pr. marinus*	0.72	0.55	1.29
		1000	1.60	0.70	2.30			1.48	0.49	3.10
		2000	1.90	0.81	2.35			1.44	0.30	4.70
		4000	2.54	0.70	3.67			2.47	0.30	8.20
Roscoff	S 6	500	0.53	0.73	0.74	Roscoff	*T. verrucosa*	0.98	0.44	2.21
		1000	1.14	0.62	1.84			1.51	0.50	3.01
		2000	1.88	0.65	2.91			2.23	0.44	5.05
		4000	2.48	0.63	3.94			2.03	0.57	3.56

decanted and measured in a spectrophotometer at 750, 665, 645, 630 and 480 mμ. Cholorphyll a content was calculated from the formula of Strickland and Parsons (1968).

OBSERVATIONS AND COMMENTS

Table I gives the data obtained from experiments on groups of 10 worms exposed to several consecutive light and dark cycles with the light held constant at 1000 foot candles. Regardless of host strain or symbiont type, the average photosynthesis/respiration value P/R, *i.e.*, (P + R)/R, remains nearly the same for all the algae. Within any group of worms, the individual P/R values for consecutive light/dark cycles generally show a progressive decrease with time. This phenomenon is also independent of symbiont type and may be analogous to the progressive decrease in photosynthetic rate observed when groups of worms are kept in the experimental chamber and are exposed to prolonged periods of light (1–1½ hr). Similar effects have been reported previously in symbiotic coelenterates and aquatic ecosystems (Beyers, 1966) and Pacific corals (Roffman, 1968). These

authors believed it to be indicative of diurnal variations in photosynthetic rate occurring under normal conditions; but specimen fatigue, photo-destruction of pigments or increased photorespiration could also account for this behavior. Any one of these latter possibilities seems to be a more likely explanation in the present case, since the time periods used here are too short to be indicative of any diurnal changes.

Table II gives typical results of data obtained from experiments relating net photosynthesis and respiration to worm volume and chlorophyll a content when the light is varied from 100 to 4000 ft-c. A different group of worms was used at each light intensity shown to avoid artifacts due to specimen fatigue, *etc.* As in previous experiments, the rate of photosynthesis with all symbionts is approximately the same at similar light values, and is independent of the host strain used. Again, minor variations in net photosynthesis with the different symbiont types, lie within the range of experimental variability. In all cases, oxygen production

Table III

Average chlorophyll a content (μg/mm³ worm)

Symbiont	Natural symbionts (*P. convolutae*)		Symbiont	Unnatural symbionts (*Prasinocladus*-type pyrenoid)	
	Worm	μg chl.a		Worm	μg chl.a
374	Roscoff	0.83 (21*)	*Prasinocladus marinus* (308)	Roscoff	0.44 (9)
	Jersey	0.65 (6)	*Prasinocladus* sp. (S 47)	Roscoff	0.43 (14)
	Hendaye	0.66 (5)	*Tetraselmis verrucosa*	Roscoff	0.44 (13)
S 6	Roscoff	0.85 (20)			
	Hendaye	0.86 (6)			
	Roscoff**	1.32 (5)			
	Hendaye**	1.10 (8)			
Hendaye	Hendaye	0.69 (19)			

* Number in parenthesis = No. of groups of 10–20 worms analyzed.
** Worms grown at low light intensity (200–300 ft-c).

increases with increasing light intensity up to the level of saturation; this occurs at or near 2000 foot candles. Dark respiration is approximately the same for all experimental symbioses except those involving *T. verrucosa* which appear to exhibit a slightly higher rate.

Important differences in the amount of chlorophyll a/mm³ worm were observed. Worms reinfected with unnatural symbionts (*Prasinocladus* types or *T. verrucosa*) have approximately $\frac{1}{2}$-$\frac{2}{3}$ the chlorophyll a of worms either bearing their natural symbionts (Hendaye/Hendaye), or reinfected with various strains of *P. convolutae* (S 6). These differences were confirmed and were evident in the average of many experiments (Table III).

As a result, if net photosynthesis is related to chlorophyll a content (Table II, last column) the unnatural symbionts produced more oxygen per unit of pigment than their natural counterparts! To account for these differences the chlorophyll a content of the various algal strains cultured under identical conditions (media

TABLE IV

Characteristics of free living symbionts in vitro

	Average Cell volume (μm^3)	μg chl.a in 10^6 cells
P. convolutae (376)	486	1.7–1.9
P. convolutae (S 6)	486	2.2–2.4
Pr. marinus (308)	1032	2.4–2.6
T. verrucosa	303	2.4
Prasinocladus sp. (S47)		2.5

and light intensity) was measured, but no significant difference was found (Table IV).

Assuming that free-living cells cultured in media have a similar amount of chlorophyll a to cells growing symbiotically, an assumption perhaps unwarranted, then the observed differences would most likely depend on the number of algal cell/worm. An examination of algal cell size, and numbers/worm confirms that this is the case. *P. convolutae* (S 6 and 374) measures 8–13 $\mu \times$ 6–10 $\mu \times$ 4–6 μ (= 192–780 μ^3) (Parke and Manton, 1967) and the average number of cells/worm observed *in situ* was 30,000 resulting in a calculated total algal volume inside the worm of 14.6×10^6 μ^3. The cells of *T. verrucosa* (*Prasinocladus*-type pyrenoid) are somewhat smaller, measuring 8–10 $\mu \times$6–6.5 $\mu \times$ 4.5–6 μ (= 216–390 μ^3) (Butcher, 1959) and are less densely packed to give an observed average of 25,000 cells/worm or a total algal volume inside the worm of 7.6×10^6 μ^3.

TABLE V

O_2 production of experimental symbionts in vitro

Foot candles	Alga	Net photo μl O_2/h	Net photo μg chlorophyll a
	P. convoluta (S 6)		
4000	3×10^5 cells*	12.3	17.1
2000	0.72 μg chlorophyll a	6.4	8.9
1000		5.3	7.4
500		4.2	5.8
	Pr. marinus (308)		
4000	1.8×10^5 cells	9.2	21.0
2000	0.43 μg chlorophyll a	5.2	12.1
1000		3.6	8.4
500		2.5	5.8
	T. verrucosa		
4000	2.5×10^5 cells	11.2	19.4
2000	0.58 μg chlorophyll a	8.2	14.2
1000		6.8	11.7
500		5.4	9.3

* Cell concentrations were selected to give the equivalent # of algae found in 10 worms (see text and Table II).

Clearly, the number of algae/worm has a direct bearing on the pigment content of animals symbiotized with *P. convolutae* and *T. verrucosa*. Among *Prasinocladus* types, the cell size of *P. marinus* (308) is larger than the natural symbiont, measuring 16–20 $\mu \times$ 7–8 $\mu \times$ 7–8 μ (= 784–1280 μ^3) (Parke and Manton, 1965), and averaged 18,000 cells/worm equivalent to an algal volume of 18.6 $\times 10^6$ μ^3. Again, the pigment content of the worms is directly related to the algal number as would be expected.

It is of interest to note that the *P. marinus* symbionts provide the host with nearly $\frac{1}{3}$ increase in the volume of algae, but only $\frac{1}{2}$ the chlorophyll a. The observations on algal number in the living worms were obtained by compressing the worms to a fixed thickness in a Rotocompressor (Biological Institute, 2018 N. Broad St., Philadelphia, Pennsylvania), and were confirmed by the apparent distribution and incidence of algal cells in thick and thin section with the electron microscope.

An examination of algal photosynthesis in culture provides a comparative basis for assessing the performance of experimental symbionts inside the host. The results of measurements on cultured symbionts are given in Table V. Substantial

Table VI

Compensation points (ft-c)

Natural symbiont (host strain + symb.)		*Unnatural symbiont* (host strain + symb.)	
Roscoff + S 6	100–150	Roscoff + 308	200–250
Roscoff + 374	100–150	Roscoff + *T. verr.*	200–250
Roscoff + W 12	100	Roscoff + S 47	100–150
Hendaye + Hendaye	100	Roscoff + S 1	250
Hendaye + S 6	200–250		
Hendaye + 374	250–300		
Jersey + 374	250		

differences in net photosynthesis exist between algae *in vitro* and in the host (*cf.* Tables II and V). This is to be expected since the host's respiration will have a direct effect on the amount of O_2 detected. Other possible contributing factors related to the conditions of a symbiotic habitat could include; (1) increased symbiont photo-respiration, and (2) a reduced or host-regulated photosynthetic rate. Comparisons between experimental symbionts based on the data in Tables II and V show that *P. convolutae* (S 6) and *T. verrucosa* maintain similar levels of O_2 exchange inside the host, *i.e.*, their net photosynthesis in the host (Table II) is approximately 22–24% of the net observed with cultures (Table V). *P. marinus* appears to be capable of maintaining a somewhat higher level of O_2 exchange, *i.e.*, net photosynthesis in the host is approximately 37% of that observed with cultures.

Discussion

This work was initiated with the hope of exploring the reasons why natural symbionts win out over unnatural ones in the competitive situation of binary rein-

fections (Provasoli *et al.,* 1968). This success might be explained if natural symbionts could be shown to establish a more effective symbiosis.

No differences in net O_2 exchange were found. P/R ratios and the values for net photosynthesis are consistently similar, regardless of host strain and symbiont type.

This similarity of results is surprising considering the striking differences in the number of symbionts per worm, and the variation in cell size among the symbiotic algae used. There are several possibilities which could explain these results.

Adaptation of the algae to low light intensities used in our cultures of *C. roscoffensis,* may be responsible for the uniform values obtained for net photosynthesis in worms with natural and unnatural symbionts. In nature, *Convoluta* inhabits sloping sand beaches where it is exposed in rivulets of drainage to full natural illumination only during low tide ($\sim$ 6 hours/day). Our cultures are maintained at 300–500 ft-c for a photoperiod of 14 hours light/10 hours dark. Despite the wholly artificial conditions, the response may be valuable in showing the great versatility of the symbiosis and its ability to grow under rather unnatural conditions. The apparent uniformity among compensation points for worms infected with a variety of experimental symbionts (Table VI) lends support to the concept of adaptation. However, intrinsic features of the symbiosis itself (see below) could also have an effect, and it seems unwise to rely too heavily on this explanation.

Inspection of electron micrographs reveals clear differences in the way in which different experimental symbionts position themselves in the animal tissues.

Low power electron micrographs show that the more numerous *P. convolutae* protoplasts are closely spaced in the worm tissue, and that they arrange themselves in 3 or more tiers in the dorsal peripheral parenchyma. Those of the *Prasinocladus* species are spaced further apart and do not arrange themselves in definite layers. This suggests that shading will be greater among the more densely packed *Platymonas* than with the loosely arranged *Prasinocladus,* and could account for the lower photosynthetic rates of *P. convolutae* inside the host.

Because respiration, with the chemical and polarographic methods, can only be measured in darkness, an important unknown is introduced in the calculation $P/R = (P + R)/R$ by assuming that dark and light respiration are equal. This assumption disregards the known differences in synthetic and metabolic events typifying the light and dark periods in synchronized cultures of single species of photosynthestic algae (Tamiya, 1966). Applying the P/R relationship to the more complex situation of a symbiosis introduces other unknowns. Are we to assume that the intimate contact between animal and vegetable cells has no influence on either? The method cannot detect variations in the rate of photorespiration of different symbionts due to the host, or variations in host respiration due to the presence and metabolic activity of different symbiont species; photosynthetic rates of symbionts may be host regulated. The uniformity of P/R obtained indicates that interdependencies between animal and vegetable cells are not apparent in the measurement of the total O_2 exchange of the symbiotic unit (host and its algae)—contrasting effects in averaging out may contribute to the uniformity.

A P/R ratio greater than 1 has been regarded lately as indication that the algae are making a positive contribution to the metabolic balance of the symbiosis (Roffman, 1968; Taylor, 1971b) because work with ^{14}C shows that radioactivity

accumulates first in the autotrophic symbionts and that the photosynthate migrates readily into animal tissues (Muscatine and Hand, 1958, review of Smith, Muscatine and Lewis, 1969 and Trench, 1971a, 1971b, 1971c).

However, PR ratios very similar to *Convoluta* have been obtained with the symbioses of corals and other marine invertebrates (Kanwisher and Wainwright, 1967; Roffman, 1968; Taylor, 1971a, 1971b). These associations include the complete spectrum of nutritional dependencies, ranging from the facultative (host feeding) symbioses of corals and *Amphiscolops* to the olbigate (host dependent on algae) symbiosis of *C. roscoffensis* examined here. Apparently, the nature of the association and its dependence or independence on an external source of food cannot be detected using P/R ratios or net photosynthesis rates.

At best, a P/R value greater than 1 can be taken to indicate only that excess photosynthate is produced, but it does not indicate its nutritional value to the animal nor that it becomes available to the animal. Muscatine (1971) found that the green algae of 2 sea anemones of the genus *Anthopleura*, photosynthesize (*i.e.*, fix carbon) but do not translocate labeled compounds to the tissue of the host, while the "normal" golden symbionts (*Gymnodinium microadriaticum*) supply their photosynthate to the same species of *Anthopleura*. The real value of the photosynthate produced cannot be assessed without biochemical and nutritional analysis.

While useful data on gas exchange in the symbiosis of *C. roscoffensis* have been obtained, they measure a mass phenomenon, therefore they cannot reveal the competitive advantage of natural symbionts in a situation of binary reinfection, which depends on more subtle and complex events. These may include unequal affinities of the animal cells to different algal species as well as the degree of interdependence and regulation between algal and animal cells.

An additional observation, the degree of intimacy between animal and algal cells, suggests a possible advantage of *P. convolutae* over the *Prasinocladus* species.

During infection, both natural and unnatural symbionts lost their flagella, eye spot and cell wall to become protoplasts (Oschman, 1966; Parke and Manton, 1967). In this condition *P. convolutae* is extremely plastic and is able to position itself between host cells in the peripheral parenchyma, establishing a network of interdigitations with both the animal cells and the muscular fibers. Obviously, the extent of host-algal contact is considerable under these conditions. In contrast, the protoplasts of *Prasinocladus* species do not interdigitate with the host cells, but remain ovate (see Plate I, Fig. 2 and Plate II, Fig. 5, Provasoli *et al.*, 1968). The interdigitation of *P. convolutae* and its greater numbers inside the worm could reasonably militate for a far higher exchange of nutrients (*i.e.*, growth) in worms reinfected with *P. convolutae* than with *Prasinocladus*. This is supported by the results of growth experiments, in which worms reinfected with *Prasinocladus* reach sexual maturity about 10 days later (Provasoli, Yamasu, Mabuchi, in press). However, extensive interdigitation has also been observed in *Platymonas* 315—a very inefficient artificial symbiont (see Plate II, Fig. 4 in Provasoli *et al.*, 1968). Newborn larvae infected with #315 have a very high mortality rate and grow to adult size in 60–100 days instead of 35–50, the usual time for *P. convoluate* and the *Prasinocladus* species-infected worms. High mortality and very slow

growth indicate that, despite extensive interdigitation, quantity and/or quality of the products released by the algae are also essential for the welfare of the host.

Summary

Measurements of oxygen balance were made with a recording polarographic electrode on 3 strains of worms reinfected with several natural and unnatural symbionts.

1. The photosynthesis/respiration (P/R) ratios and net photosynthesis varied with light intensity but were independent of worm strains or algal species; compensation light intensities were also similar.

2. However, the content in chlorophyll a/mm^3 of worm varied and in worms reinfected with unnatural symbionts was $\frac{1}{3}$–$\frac{1}{2}$ that found in natural symbiont reinfected worm. Consequently, worms with unnatural symbionts produced more $O_2/\mu g$ chlorophyll a.

3. Cell size of different species of symbiont varied but average chl. a/cell was similar. Observations *in vivo* and with thick and thin EM sections showed that the number of symbionts/mm^3 worm varied with symbiont species. Natural symbionts were more numerous, and more densely packed in superimposed tiers, mutual shading may be responsible for a less efficient photosynthesis.

4. However, adaptation to laboratory light intensities and the assumption implicit in the measurements and calculations of the P/R that dark respiration is equal to photorespiration may also account for the uniformity of results with different symbionts.

5. The P/R ratios appear to be an insensitive way to distinguish obligatory from faculitative symbionts. At best, positive P/R ratios indicate that excess photosynthate is produced but it does not indicate whether or not the photosynthate is released, taken up and/or utilized by the host, nor its nutritional contribution.

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